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Development of Pantothenate Analogs That Can Treat Combat-Related Infections

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INTRODUCTION:

A goal of the studies is to develop antimicrobial compounds with novel modes of action to treat the battlefield infections from *Staphylococcus aureus*, *Klebsiella pneumoniae* and extended-spectrum beta-lactamase enterobacteriaceae including *E. coli*. N-substituted pantothenate analogs have been developed and shown to inhibit the growth of *S. aureus* and *E. coli* (and probably also *K. pneumoniae*) but have not been tested in preclinical and clinical set-ups, primarily because of their possible interference with human cells. We propose to elucidate differences in the architecture of the compound-pantothenate kinase binding site between humans and bacteria using x-ray crystallographic techniques and exploit these differences to develop new compounds specific for the drug-resistant bacterial strains.

BODY:

In 2011. We reported the crystal structures of *S. aureus* PanK (SaPanK) in complex with N-pentyl and heptyl pantothenate analogs (i.e., N5-Pan and N7-Pan, called the 1st generation Pan analogs), *K. pneumoniae* PanK (KpPanK) in complex with N5-Pan, *E. coli* PanK (EcPanK) in complex with N5-Pan and human PanK3 (hPanK3) in complex with N7-Pan. We also reported that N5-Pan and N7-Pan had minimal inhibitory concentrations (MICs) of lower than 10 μg/ml for *S. aureus* ATCC 29213. Finally, we reported that N5-Pan and N7-Pan as well as two newly synthesized compounds (i.e., MT-183 and MT-190, called the 2nd generation Pan analogs) showed no cytotoxicity to HepG2 liver cells. Similarly to that to HepG2 liver cells, N5-Pan and N7-Pan showed no cytotoxicity to A549 lung cells.

Determination of minimal inhibitory concentrations by broth microdilution. For assessing the bacterial-killing capability of the newly synthesized compounds of MT-183 and MT-190, we determined their MICs against S. aureus ATCC 29213, K. pneumoniae ATCC 10031 and E. coli ATCC 25922 by using a broth microdilution assay. Briefly, bacterial cultures were prepared by inoculating 5 ml of Laura broth with a small amount of the desired glycerol stock of each bacterial strain and allowed to grow at 37°C to an A_{600} of ~0.1. 96-Well plates were prepared containing 50 ul of two-fold serial dilutions (20 mg/ml~0.00976 mg/ml) of the new compounds and then inoculated with another 50 ul of the desired culture (the desired final inoculum size is 5×10^5 cfu/ml) and allowed to incubate at 37°C for 18 h. The optical density was measured for all cultures at A₆₀₀, and the MIC was determined from the concentration of Pan analogs at which no growth was observed. All MIC determinations were performed in duplicate. The MIC results are in Table 1. MT-190 showed the MIC of 250 µg/ml for S. aureus and higher MICs (>2.0 mg/ml) for E. coli and P. pneumoniae. MT-183 showed the MIC of 1 mg/ml for P. pneumoniae and higher MICs (>2.0 mg/ml) for E. coli and S. aureus. These findings suggest some degree selectivity of MT-190 toward S. aureus and MT-183 toward P. pneumoniae, indicating that these compounds merit further study as part of a antimicrobial drug discovery project.

Table 1. **Minimum Inhibitory Concentrations of MT-0183 and MT-0190 Pan analogs.** Cefoxitin was used as a control for *E. coli and K. pneumoniae* whereas gentamicin for *S. aureus*.

Strains	Compounds (mg/ml)			
	MT-0183	MT-0190	Cefoxitin	Gentamicin
E.coli	> 2	> 2	0.008	-
K.pneumoniae	1	> 2	< 0.001	-
S.aureus	> 2	0.25	-	0.008

Structural validation for the binding of the 2nd generation Pan analogs to PanKs

To achieve optimal lead compounds from MT-190, we solved the co-crystal structure of SaPanK with MT-190 (Fig. 1). Diffraction data was collected at 1.8 Å and the current structure has the working R-factor value of 24% and the free R-factor of 28%. The hydrophobic portions of several residues (Leu171, Thr172, Glu202, Val236 and Tyr240) form a non-polar pocket for the aromatic moiety of MT-190 (Fig. 1). Please notice that the same residues are involved in forming the binding pocket for N7-Pan (data not shown). Importantly, the aromatic moiety of MT-0190 is inserted in between the side chains of Glu202 and Tyr240 and the side chain of Glu202 is stabilized by that of Thr172 via a hydrogen bond (Fig. 1). More importantly, there is a space between the aromatic moiety of MT-190 and Val236, which can direct the structural modifications of MT-190 specific for SaPanK.

In addition, we solved the structure of the KpPanK in complex with MT-183 at 2.0Å resolution and the current structure has the working R-factor value of 22% and the free R-factor of 26%. In the structure, the nitrogen-substituted benzyl moiety of MT-183 is surrounded by multiple non-polar residues providing hydrophobic contacts (left panel in Fig. 2). A surprising observation is that the benzyl moiety can be bound at the active site in two alternative conformations (right panel in Fig. 2). This finding suggests that a branched compound (e.g., a substituent with two benzyl moieties) may improve binding potency toward KpPanK.

Figure 1. **The MT-190 binding site of SaPanK**. *Left,* newly synthesized Pan analogs are shown. *Middle,* MT-0190 (magenta, stick) is bound to both the molecules of SaPank dimer (green and cyan). A zoomed-in view of the MT-0190 binding site shown on the right panel. *Right,* the aromatic moiety of MT-0190 (magenta, carbon) interacts with hydrophobic portions of several residues, L171, T172, E202, V236 and Y240. Dotted line indicates a hydrogen bond between T172 and E202.

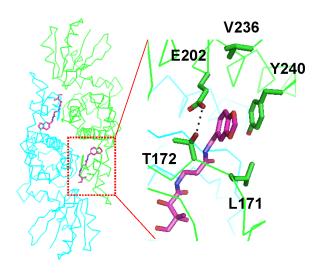
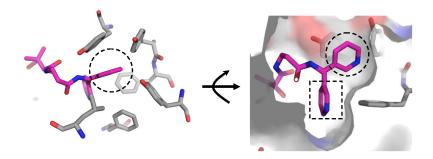


Figure 2. The MT-183 binding pocket of KpPanK. Left panel, a view showing the benzyl moiety interactions with hydrophobic residues of KpPanK (Y180, F244, Y258, F259 and Y262). Dotted circle indicates the benzyl moiety. Right panel, a view with a horizontal rotation of 90 degrees from the left panel. Dotted circle indicates the same benzyl moiety as in the left panel whereas dotted rectangle shows an alternative binding conformation of the benzyl moiety supported by electron density (data not shown).



The 3rd generation Pan analogs based on the co-crystal structures of the SaPanK-MT190 and the KpPanK-MT183 complexes.

We designed a 3rd generation chemical series against SaPanK by utilizing the MT-190 bound structure of SaPanK and the MT-183 bound structure of KpPanK (Fig. 3). These new compounds were characterized using bacterial colony and human cell cytotoxicity assays (see below)

Figure 3. The 3rd generation Pan analogs including the earlier generation Pan analogs.

<u>Determination of minimal inhibitory concentrations of the 3rd generation Pan analogs by agar</u> diffusion

To screen the bacteria-killing capability of 3rd generation Pan analogs, we used the agar diffusion method against *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10031. Bacterial cultures were prepared by inoculating 3 ml of tryptic soy broth with the small amount of bacteria from a glycerol stock, and allowed them to grow at 37°C. The cultured bacterial cells were spread onto a muller hinton and tryptic soy agar plate and each Pan analog dispensed on a disk was placed on the plate. The plates were allowed to incubate at 37°C for 24 h. Six compounds (MT-348, -352, -354, -355, -356 and -358) inhibited the growth of *S. aureus*, whereas three compounds (MT-348, -354 and -356) were effective against *K. pneumoniae*. For *E. coli*, however, no compounds had any killing effect. We will further quantitate these effective compounds by measuring the MIC values using broth microdilution. Please notice that the compounds (MT-354, -355 and -358) will be excluded from the MIC assay due to their significant cytotoxicity to HepG2 cells (see below).

Determination of cytotoxicity of the 3rd generation Pan analogs by the MTT assay

Eleven 3rd generation Pan analogs and Hopantenate (Ho-Pan) were tested against human HepG2 hepatocellular carcinoma cells to assess their cytotoxicity. The HepG2 cell line was purchased from ATCC (ATCC HB-8065) and cultured in complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine and 1% Penicillin/Streptomycin. The cells were grown in 37°C incubator with 5% CO₂ in air atmosphere for 48 hours until 80% cell confluence was achieved. The cells were then washed with DPBS and trypsinized to be detached from the culture flask and reequilibrated in fresh culture medium.

After the 48 hours incubation of the cells with compounds dissolved in 1% DMSO, cell viability was determined by MTT assay. First, cell media was discarded and the cells were incubated in 10% MTT dye (Thiazolyl blue tetrazolium bromide) for 2.5 hours in 37°C incubator with 5% CO₂ in air atmosphere. Then, DMSO was added to each well to solubilize the MTT dye. The absorbance was measured at the wavelength of 570 nm and corrected by the background wavelength of 650 nm. The 1% DMSO control solution was used to normalize data. A sigmoidal concentration-response curve was generated using analysis software (Prism-Graphpad, ver. 6), calculating the IC₅₀ value of each tested compound.

The concentration-response curve was constructed to assess cytotoxicity of the tested compounds by MTT assay after 48 hours incubation. Compounds MT-348, -350, -351, -352, -356, -357 and Ho-Pan, as well as MT-182, -183 and -190 from previous generation, were not significantly cytotoxic to human HepG2 hepatocellular carcinoma cells up to the concentration of 2.5mM (Table 2). On the other hand, compounds MT-181, -349, -353 and -354 showed cytotoxic potential with IC_{50} value of 1mM. Compounds MT-355 and -358 were considerably cytotoxic to HepG2 cells with the IC_{50} value of 0.4mM (Table 1).

Table 2	IC ₅₀	values	of Pan	K com	pounds.
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Chemical	IC ₅₀	Chemical	IC ₅₀	Chemical	IC ₅₀	Chemical	IC_{50}
Structure &	(mM)	Structure &	(mM)	Structure &	(mM)	Structure &	(mM)
Name		Name		Name		Name	
SGC-DS-MT-0181-b1/b2 HO 0 316.44 0 NH OH NH	1.1	SGC-DS-MT-0348-b1 O 338-40 O NH NH NH OMe	N/A	SGC-DS-MT-0352-b1 HO	N/A	SGC-DS-MT-0356-b1 O 314.42 O NH NH NH	N/A
MT-181 (N7)		MT-348		MT-352		MT-356	
SGC-DS-MT-0182-b1/b2/b3 O 288.38 O NH NH NH	N/A	SGC-DS-MT-0349-b1 384.47 HO NH NH	1.3	SGC-DS-MT-0353-b1 O 396.48 O OMe OH OH	1.1	SGC-DS-MT-0357-b1 HO 0 368.42 O OMe OH MeO	N/A
MT-182 (N5)		MT-349		MT-353		MT-357	
SGC-DS-MT-0183-b1 OH OH NH NH NH NH	N/A	SGC-DS-MT-0350-b1 HO	N/A	SGC-DS-MT-0354-b1 O 366.41 O NH NH O	1.4	SGC-DS-MT-0358-b1 0 398.50 0 HO OH NH NH	0.4
MT-183		MT-350		MT354		MT-358	
SGC-DS-MT-0190-b1/b2 O 352.38 O NH NH NH O	N/A	SGC-DS-MT-0351-b1 O 398.45 O HO	N/A	SQC-DS-MT-0355-b1 HO NH NH NH	0.4		N/A
MT-190		MT-351		MT-355		Ho-Pan	

KEY RESEARCH ACCOMPLISHMENTS:

- The minimal inhibitory concentration of MT-190 against S. aureus was \sim 250 μ g/ml, which was somewhat selective to S. aureus
- The minimal inhibitory concentration of MT-183 against *K. pneumoniae* was 1 mg/ml, which was somewhat selective to *K. pneumoniae*
- Determination of the MT-190 bound structure of *S. aureus* PanK
- Determination of the MT-183 bound structure of *K. pneumoniae* PanK

• Synthesis of 3rd generation Pan analogs based on the MT-183 and MT-190 bound PanK structures, several of which showed the effective killing against *S. aureus* (i.e., MT-348, -352 and -356) and *K. pneumoniae* (i.e., MT-348 and -356) without cytotoxicity to HepG2 liver cells.

REPORTABLE OUTCOMES:

A manuscript entitled "Crystal structures of *Klebsiella pneumoniae* pantothenate kinase in complex with N-substituted pantothenamides" has been accepted with modification for publication at Proteins: Structure, Function, and Bioinformatics.

CONCLUSION:

We have solved the co-crystal structures of SaPanK with MT-190 and KpPanK with MT-183. Based on these structures, we have synthesized 11 new compounds that fit to the Pan analog binding site of SaPanK or KpPanK. We have showed qualitative bacterial killing efficiency of these 11 compounds by agar diffusion and their cytotoxicity to HepG2 cells and plan to perform the quantitative measure of bacterial killing using broth microdilution. These new compounds will be the basis for further optimization to develop a novel class of narrow-spectrum antibiotics against the multidrug resistant strains of *S. aureus* and *K. pneumoniae*.

REFERENCES:

n/a

APPENDICES:

Abstract for the manuscript at Proteins: Structure, Function, and Bioinformatics

N-substituted pantothenamides are derivatives of pantothenate, the precursor in the biosynthesis of the essential metabolic cofactor coenzyme A (CoA). These compounds are substrates of pantothenate kinase (PanK) in the first step of CoA biosynthesis and possess antimicrobial activity against various pathogenic bacteria. Here we solved the crystal structure of the *Klebsiella pneumoniae* PanK (KpPanK) in complex with N-pentylpantothenamide (N5-Pan) to understand the molecular basis of its antimicrobial activity. The structure reveals a polar pocket interacting with the pantothenate moiety of N5-Pan and an aromatic pocket loosely protecting the pentyl tail, suggesting that the introduction of an aromatic ring to a new pantothenamide may enhance its binding affinity to KpPanK. To test this idea, we synthesized N-pyridin-3-ylmethylpantothenamide (Np-Pan) and solved its co-crystal structure with KpPanK. The structure reveals two alternative conformations of the aromatic ring of Np-Pan bound at the aromatic pocket, providing the basis for further improvement of pantothenamide binding to KpPanK.